IMMUNOLOGICAL METHOD

The present invention relates to an immunological method, in particular it relates to a method of identifying antigen-responsive T cells.

T cells are fundamental to the immune process. They play a central role as either regulator or effector in a wide range of immune-driven responses. Their function can be beneficial to the host as in the immune response to infections or tumours but it may also be detrimental such as in autoimmunity, allergy and transplant rejection.

Unambiguous identification of the T cells responsible for these responses would aid the development of vaccines to infections and perhaps tumours and would allow immunosuppressive therapy to be specifically targeted in those diseases where T cell overactivity is pathogenic.

T cells recognise peptide antigens presented to them in the context of major-histocompatibility complex-encoded molecules [1,2]. They accomplish this by virtue of a cell-surface, clonally-distributed heterodimer known as the idiotypic T cell receptor (TCR). The TCR is usually composed of an α and β chain and more rarely a γ and δ chain. Each of these chains has an immunoglobulin-like structure with a variable and a constant domain. The constant domain (as its name suggests) is the same within each chain type (ie all α chain constant regions are identical) but the variable domain differs between each TCR [1,3-5].

The diverse range of variable domain structures arise because the gene which encodes them is formed by the random recombination of smaller gene segments which are imprecisely joined together. These smaller segments are known as variable (V), diversity (D) (β and δ chains only) and joining (J) gene segments [6]. Studies of the structure of the chromosomes which encode the TCR chains reveal there are 50-52 functional *TCRBV* (β chain variable gene segments) [7], at least 70 *TCRAV* (α chain variable gene segments) [8-14] and 57 *TCRAJ* (α chain joining gene segments) [15]. Given the numbers of these smaller gene segments, their random recombination and the imprecise mechanism which creates more diversity when they are joined, the TCR repertoire has been estimated to be between 10^{15} and 10^{18} different receptors even when inadvertent stop codons or out-of-frame redundancy is taken into account [16-18]. The complete 685-kilobase DNA sequence of the human β T cell receptor locus is known (Rowen *et al* (1996) *Science* **272**, 1755-1762). The sequence and its annotations are deposited in the Genome Sequence Data Base with accession numbers L36092, L36190 and U03115.

Identification of antigen responsive T cells is relatively easy when either the stimulating antigen or the responding TCR structure is known. Unfortunately, in the majority of clinical situations neither is known.

Methods purporting to identify antigen-responsive T cells have been described and can be broadly classified as "immunohistochemistry" and "molecular biology" approaches.

Two different immunochemistry approaches have been used. The first involves the use of immunohistochemical techniques to enumerate the numbers of T cells bearing particular *TCRBV*-encoded gene products and to establish how many of these cells bear so-called activation markers (eg HLA-DR, CD25). All these techniques are presumed to measure the repertoire of T cells within an immune lesion. It is assumed that as the T cells responding to antigen proliferate, the most numerous T cell (in comparison to controls either anatomic or disease-based) must be the one driven to division by antigen.

I believe that this reasoning appears to be flawed. It is known that only a small percentage of T cells in any immune lesion are antigen specific [19-21]. Others are passively recruited there. CD45RO (memory) T cells preferentially home to sites of inflammation [22-24], so the repertoire of T cells in a lesion is not only determined by the appropriate antigen but the antigens which have induced memory T cells in the past. The range of previous immunological experience is almost impossible to take into account adequately and is one reason for the variability of results obtained by different workers. Another problem that dogs this approach is that while molecules like HLA-DR and CD25 are undoubtedly expressed on active T cells they are not necessarily induced by a specific encounter with antigen. Cell-surface expression of CD25 can be induced by interleukin-2 (IL-2) produced in either autocrine or paracrine fashion [25,26] as well as other cytokines such as IL-7 [27,28], tumour necrosis factor [29] or T-cell reactive ligands such as CD40L [30]; HLA-DR can be similarly upregulated by interferon-γ [31]. In other words, in active immune lesions there are cytokines which can induce 'activation molecules' on T cells without them being

directly stimulated by antigen. It is therefore impossible to surmise which T cell is being triggered by antigen and which passively activated by chemokines using this methodology.

The molecular biological approach involves enumerating the *spectrum* of TCR mRNAs expressed at a particular site or in a particular lesion and comparing this to the TCR mRNA repertoire at other sites or in control individuals. A variety of techniques have been employed to accomplish this which are mostly dependent on the polymerase chain reaction (PCR) [32]. The most reliable techniques in terms of enumeration have been inverse PCR or anchored PCR. Other semi-quantitative techniques such as family-specific PCR have also been used. This approach is essentially similar to immunohistochemical one but, since all the *TCRBV* genes are now known, it is more complete than has been achievable to date with a more limited range of anti-Vβ monoclonal antibodies.

It has been assumed previously that TCR mRNA levels approximate to specific T cell numbers but in order for this to be true two basic assumptions must also be correct: (1) all TCR genes should be transcribed at the same rate; and (2) gene transcription does not vary when T cells are stimulated. As discussed in more detail below, my data suggest that both of these assumptions are incorrect.

The TCR can show exquisite specificity for peptide antigen presented in the context of MHC molecules, yet the affinity of the TCR for the peptide/MHC complex is low and the off rate for the interaction is high [33-35]. Further complicating this apparent paradox is the fact that as few as 100 peptide/MHC complexes may be required to fully trigger

specific T cells, and the fact that sustained TCR to peptide/MHC complex contact is required for full T cell commitment to activation [36-38].

Recent data suggests that the T cell uses its cytoskeleton to move over the surface of the antigen-presenting cell, sequentially making contact with relatively few of the antigen-presenting cell's [APC's] MHC/peptide complexes, with tens of thousands of its own surface TCRs [39]. The summation of each of these signals, over a period of time, leads to the sustained second messenger levels required for a commitment to T cell activation [38-40].

Usually, there is a constant recycling of cell surface TCRs [41,42] but after phosphorylation following antigen-triggering the receptors are internalised and degraded [43-45]. Studies using T cell clones which fortuitously have two separate TCRs have shown that the receptor binding antigen is downregulated from the cell surface, while the non-reactive TCR remains at an unaltered surface density [46].

Semi-quantitative measurement of *TCRBV2* mRNA levels in patients suffering from toxic-shock syndrome showed that mRNA levels increased sharply during the acute phase of the illness and settled to control levels within approximately three months of this condition being successfully treated. The toxic shock syndrome toxin (TSST-1) is a superantigen specific for *TCRBV2*-encoded TCRs [47,48]. The TCR Vβ mRNA is not measured on a "per specific T-cell" basis. In Kawasaki disease, a condition caused by *Staphylococci* and *Streptococci* releasing a TSST-1-like superantigen, the rate of production of *TCRBV2S1* mRNA

by individual T cells increased in the acute phase of the disease and settled to control levels after treatment. By contrast, in the same work, *TCRBV12* mRNA production rates were shown not to alter after T cells were treated with SEB (a different *TCRBV12*-specific superantigen) *in vitro*. However, although the mRNA production rate appeared constant both cell numbers and mRNA levels increased proportionally after this treatment [49,50]. There is no suggestion that measurement of the increase in specific TCR mRNA production per specific T cell can be used generally to identify antigen-response T cells when the stimulating antigen and responding TCR are not known.

Duchmann *et al* (1993) *DNA and Cell Biol.* **12**, 217-225 describes a purportedly quantitative method for measuring TCR Vβ subfamilies by reverse transcriptase (RT)-PCR, but TCR mRNA is not measured per specific T cell.

There remains the need for a method of identifying antigen responsive T cells (or the particular TCR involved in an antigen response) particularly in the case when the stimulating antigen and the responding TCR are not known.

One object of the present invention is to provide a method which allows the identification of antigen responsive T cells or the particular TCR involved in an antigen response when the stimulating antigen is not known and when there is no clue or few clues as to what particular T cell or TCR is involved in an antigen response.

The method is particularly useful for identifying T cell (and T-cell receptor; TCR) types involved in antigen-mediated diseases. Many human diseases are believed to involve antigen-driven T cells including allergies, autoimmune disease, allograft rejection and acceptance, some infectious diseases such as parasitic diseases, and some cancers. These diseases include, for example, multiple sclerosis, farmer's lung, hay-fever and eczema.

I propose that TCR gene expression is increased after antigen stimulation in order to replace the receptors which have been lost from the cell surface during antigen triggering. As published data suggests that almost half of the cell surface TCRs are required to bind antigen (and are therefore phosphorylated and internalised) to commit the T cell to full activation [46], I propose that this mechanism may be vital for normal T cell function.

As T cell activation by cytokines would not involve degradation of cell-surface TCRs, I propose that measurement of TCR-specific mRNA production rates is a particularly suitable method to discriminate between passively recruited/passively-activated T cells and antigen-specific T cell effectors in any immune process. Typically, the rate of mRNA synthesis is measured over a fixed period (ie there is a fixed time between antigen contact with the T cell and the time when the mRNA is measured) and so the rate of mRNA production is equivalent to the amount of mRNA synthesised in a fixed period of time. Chronic diseases involving antigen-mediated processes are believed to involve chronic presentation of antigen to T cells. In these circumstances it can

reasonably be assumed that T cells are constantly being triggered by antigen.

In the preferred embodiment of the method of the invention the measurement of specific TCR mRNA production per specific T cell indicates antigen stimulation and not the measurement of either specific T cell numbers or mRNA alone. In clinical situations, where massive T cell activation occurs (for example in toxic shock syndrome), quantitation of either specific cells or mRNA may be sufficient to make a determination of the T cell responsive to a particular antigen (although it helps to know which T cell receptor subset (such as a particular Vβ) and which superantigen you are looking for). However, these methods (ie measuring specific T cell numbers or measuring specific T cell receptor mRNA) will not identify antigen-triggered cells in more subtle situations or when conventionally-processed antigens (the vast majority) are responsible.

A first aspect of the invention provides a method of identifying an antigen-responsive T cell within a population of T cells, the method comprising the steps of

(1) obtaining a sample containing T cells which have responded to the antigen;

(2) determining individually for each of a plurality of specific T cell receptors, or individually for each of a plurality of subsets of T cell receptors, whether expression of a gene encoding a specific T cell receptor, or whether expression of genes encoding a subset of

T cell receptors, has increased per specific T cell receptor-positive T cell or per specific T cell receptor-positive T cell subset compared to the expression of said gene or genes in a sample containing T cells which have not responded to the antigen.

The increase in specific TCR gene expression following antigen stimulation may be determined using any suitable method. Typically, following TCR gene expression, mRNA is synthesised and the mRNA is translated into polypeptide. Any protocol for identifying mRNA synthesis may be used and, since mRNA is relatively unstable, measurements of the amount of mRNA over a particular time period is probably a reasonable estimate of the synthesis of new mRNA. Polypeptides are generally more stable than mRNA and so, typically, measuring the amount of specific TCR polypeptide may not distinguish between existing TCR polypeptide and newly synthesised TCR polypeptide. Methods which can distinguish newly synthesised, specific TCR polypeptides and existing, specific TCR polypeptides may, however, be used in the method of the invention but it is preferred if mRNA specific for an individual T cell receptors or for subsets of T cell receptors is measured.

The sample containing T cells which have not responded to the antigen may be any suitable control sample as is discussed in more detail below. The control level of expression of a gene encoding a specific T cell receptor or of genes encoding a subset of T cell receptors is measured as for the test sample and, typically, a control level can be set for each specific T cell receptor or subset of T cell receptors. Thus, in one

embodiment of the invention the comparison of the level of specific gene expression in a sample which has responded to the antigen with a level in a sample containing T cells which have not responded to the antigen may be an historic comparison with the levels in a control sample which has been determined separately at an earlier time although, of course, it is particularly preferred if a substantially identical protocol has been used to measure the level of gene expression (eg the amounts of specific T cell receptor mRNA) in the test sample and the control sample.

Alternatively, and still preferably, as is discussed below, the comparison of the levels of gene expression may be measured in samples taken and, optionally, analysed contemporaneously.

A particularly preferred embodiment of the invention provides a method of identifying an antigen-responsive T cell within a population of T cells, the method comprising the steps of

(1) obtaining a sample containing T cells which have responded to the antigen;

(2) determining individually for each of a plurality of specific T cell receptors, or individually for each of a plurality of subsets of T cell receptors, the amount of T cell receptor mRNA, which mRNA is specific for a T cell receptor or is specific for a subset of T cell receptors, per specific T cell receptor-positive T cell or per specific T cell receptor-positive T cell subset, in the sample obtained in step (1); and

(3) determining which T cell receptor mRNA has an increased amount per specific T cell in the samples obtained in step (1) compared to that in a sample containing T cells which have not responded to the antigen.

In one preferred embodiment step (1) comprises obtaining (a) a sample containing T cells which have not responded to the antigen and (b) a sample containing T cells which have responded to the antigen and in step (3) it is determined which T cell receptor mRNA has an increased amount per specific T cell in sample (b) compared to sample (a).

Thus, it can be seen that in some circumstances samples (a) and (b) can be taken and, optionally, the specific TCR mRNA measured, contemporaneously or in some circumstances sample (a) may be an historic test sample.

Conveniently, normal ranges of specific TCR gene expression for untriggered T cells (ie those which have not responded to antigen) can be obtained by reference to normal, healthy individuals. For example, T cells can be obtained by bleeding a suitable number (eg 8 to 20) normal healthy individuals and measuring the numbers of TCR mRNA molecules per T cell for each specific TCR gene or specific subset of TCR genes that one wishes to study.

It can be seen that, for example, in the four individuals studied in Example 1, the person-to-person variation in the numbers of TCR-specific mRNA molecules per cell is not great for both *TCRBV2S1* and *TCRBV3S1* (Figures 4 and 5). It should be noted that there is little

variation in the numbers of specific TCR mRNA molecules per cell between T cells freshly obtained from subjects' peripheral blood (PRE sample) and the same cells cultured for three days in the presence of RPMI1640 and 10% heat-inactivated foetal calf serum (CON sample) as described in Example 1. Thus, the control sample may be from suitable T cells in culture.

It is also useful to establish ranges of expression for antigen-triggered T cells. Ranges for numbers of specific TCR mRNA molecules per cell in antigen-triggered T cells can be derived, in culture, by, for example, triggering the T cells with either superantigens or anti-Vβ-specific monoclonal antibodies or anti-CD3 antibodies. The last two methods of triggering are considered to approximate to the situation encountered with conventional antigen *in vitro*. Once again, if a suitable number (eg 8 to 20) of normal individuals are used as a source of T cells, a range for triggered TCR mRNA molecules per cell can be obtained.

Ranges for triggered TCR mRNA per cell levels may be obtained *in vivo* if a number of patients suffering with TCRBV-specific diseases are studied during the course of their disease and then again once they had successfully recovered. Examples, as discussed in more detail below, include *TCRBV2S1* mRNA levels per T cell in Toxic shock syndrome or Kawasaki disease.

The method of the invention may be used to identify a specific antigen-responsive T cell or it may be used to identify a subset of T cells involved in a particular antigen response; for example, the method may be used to identify a T cell receptor subset each of which contain a

common Vβ segment or a common Vα segment or combinations thereof. However, it will be appreciated that smaller and smaller subsets of T cells involved in an antigen response can be identified by, for example, first identifying the specific Vβ subset and then the specific Vβ-J subset and then the specific Vα subset and then the specific Vα-J subset and then the specific Vβ-J/Vα-J subset of TCR.

The method is particularly useful in identifying an antigen-responsive T cell, and therefore a specific T cell receptor type, which is associated with a disease state. The population of T cells may be any suitable population of T cells, for example a population of T cells from a mammal. It is preferred if the population of T cells is a population from a human patient; in particular it is preferred if the population of T cells is a population of T cells associated with a disease in a human patient.

Conveniently, the sample containing T cells which have not responded to the antigen ("sample (a)") is obtained from a non-diseased site of an individual and the sample containing T cells which have responded to the antigen ("sample (b)") is obtained from a diseased site of an individual. For example, sample (a) may be obtained from non-diseased synovial samples of a patient whereas sample (b) may be obtained from synovial samples of joints showing signs of rheumatoid arthritis from the same patient. In this way, using the method of the invention antigen-responsive T cells involved in rheumatoid arthritis may be identified.

Also conveniently, the sample containing T cells which have not responded to the antigen ("sample (a)") is obtained from a non-diseased individual and the sample containing T cells which have responded to the

antigen ("sample (b)") is obtained from a diseased individual. For example, sample (a) may be obtained from a healthy control individual or a convalescent Kawasaki disease patient and sample (b) may be obtained from an acute Kawasaki disease patient.

It will be appreciated that in some circumstances the sample containing T cells which have responded to antigen is a sample obtained from an individual which has been contacted with the antigen (which may be in the form of a disease-causing agent) *in vitro*. For example, the sample may be a T-cell-containing sample from an individual which is treated with a microorganism *in vitro* or which is treated *in vivo* with an antigen or mixture of antigens derived from a microorganism. For example, the population of T cells may be lymphocytes obtained from a peripheral blood sample which are treated with *Staphylococcal enterotoxin* B (SEB; a superantigen).

Although the method may be used to identify an antigen-responsive T cell within a population when the disease-associated antigen is known, it is particularly preferred if the method is used to identify an antigen-responsive T cell within a population of T cells when the antigen or antigens associated with a disease is not known. The method may be used to identify a T cell responsive to a superantigen but it is preferred if it is used to identify a T cell responsive to a conventional antigen.

Superantigens are typically the protein products of a number of bacteria and viruses. Their name derives from their ability to stimulate large numbers of T cells compared with that seen with conventional antigens. They differ from conventional antigens in a number of ways:

Superantigens function as intact proteins. Conventional antigens are generally small peptides, of between 8 and 12 amino acids long, derived from the internalisation and proteolytic degradation of larger proteins by an antigen-presenting cell.

Superantigens bind outside the 'peptide binding groove' of the major histocompatibility complex class II molecule on the surface of antigen-presenting cells. Conventional antigenic peptides lie in this groove in the class II molecule.

Superantigens generally bind to a specific region of the β chain of T cell receptor called the fourth hypervariable region. This is encoded by the *TCRBV* gene segment alone. The structure of the TCR α chain and the *TCRBJ* gene segment may play a minor role in influencing the affinity of superantigen binding. Conventional antigens are thought to be recognised by the complementarity determining regions (especially CDR3 which is formed by the combination of V-(D)-J gene segment recombination and N region additions) of both the α and β TCR chains. (See Kay R.A. (1995) *Clin. Exp. Immunol.* **100**, 4-6; and Herman A., *et al* (1991) *Annu. Rev. Immunol.* **9**, 745-772.)

It will be appreciated that for many diseases there may be more than one disease-associated antigen and that therefore there may be more than one antigen-responsive T cell type within a population of T cells. The method is believed to be useful in identifying each antigen-responsive T cell corresponding to a disease-associated antigen but it will be appreciated that the method is particularly suited to identifying antigen-

responsive T cells where the specific T cell receptor mRNA production per specific T cell is highest.

It will be appreciated that the samples containing T cells may be any suitable samples containing T cells. Conveniently, the sample is a sample of peripheral blood or a sample of bone marrow but it may be any sample from an individual which contains T cells. Samples from an individual which are then cultivated *in vitro* may also be used.

In a preferred embodiment of the invention the subset of T cell receptors is a subset wherein each T cell receptor comprises a specific Vβ region or segment. The specific Vβ region or segment of the TCR mRNA may be recognised using a specific nucleic acid probe which hybridises to the specific Vβ segment mRNA. Any convenient method for identifying and quantitating the amount of mRNA containing a specific Vβ segment may be used, for example "chip" hybridisation methods of detecting specific mRNA or cDNA may be used. As is described in more detail below it is particularly preferred in the polymerase chain reaction (PCR) is used; more particularly it is preferred if a quantitative PCR method is used. It will be appreciated that since PCR relies on a DNA template the TCR mRNA should be copied into cDNA prior to or during the PCR process.

Methods for synthesising cDNA from mRNA are well known in the art and typically involve hybridising an oligonucleotide primer to the mRNA and synthesising DNA using deoxynucleotides and a reverse transcriptase. Methods for performing polymerase chain reactions are well known in the art. Methods of cDNA synthesis and PCR methods

are described in Sambrook *et al* (1989) *Molecular cloning, a laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory, New York, incorporated herein by reference.

In a further preferred embodiment of the invention the subset of T cell receptors is a subset wherein each T cell receptor comprises a specific Vα region or segment. The specific Vα region of the TCR mRNA may be recognised using a specific nucleic acid probe which hybridises to the specific Vα segment mRNA. Similar methods of identifying and quantitating the specific Vα segment-containing TCR mRNA may be used to those for identifying and quantitating the specific Vβ segment-containing TCR mRNA.

Nucleotide sequence information is available for many of the β chain variable gene segments (TCRBV) and α chain variable gene segments (TCRAV) and α chain joining gene segments (TCRAJ) as well as for other segments of the TCR genes which are transcribed and spliced into TCR mRNA. Much of this information is available from publicly accessible nucleotide sequence databases such as Gen Bank and EMBL. In particular, the complete 685-kb DNA sequence of the human β T cell receptor locus is known (Rowen *et al* (1996) *Science* **272**, 1755-1762). The sequence and its annotations are deposited in the Genome Sequence Data Base with accession numbers L36092, L36190 and U03115, all incorporated herein by reference. Suitable probes and primers for measuring specific Vβ, Vα and other TCR gene segments are readily derived from the publicly available sequences for the TCR genes.

It is preferred if the amount of T cell receptor mRNA is determined using quantitative PCR; and it is particularly preferred if the quantitative PCR method is reverse-transcription competitive PCR (RT-CPCR). Reverse-transcription competitive PCR (RT-CPCR) is described in detail in Kohsaka *et al* (1993) *Nucl. Acids Res.* **21**, 3469-3472 and in Taniguchi *et al* (1994) *J. Immunol. Methods* **169**, 101-109, both of which are incorporated herein by reference.

For the measurement of any particular TCR gene segment by PCR at least one TCR gene segment-specific oligonucleotide primer is required. Although quantitation of specific TCR gene segment-containing mRNA may be carried out using a pair of PCR primers each of which hybridise within the specific TCR gene segment, it is convenient if one of the primers hybridises within the specific TCR gene segment and the other primer hybridises to a segment of TCR mRNA (cDNA) adjacent in the mRNA (cDNA) but which is separated (for example by an intron) in the genomic DNA. This approach may substantially prevent amplification of any contaminating genomic DNA; and, therefore, the method is useful for discriminating between TCR cDNA/mRNA and TCR genomic DNA.

Suitable PCR primers for identifying specific Vα and Vβ segments in TCR mRNA are described in Williams *et al* (1992) *J. Clin. Invest.* **90**, 326-333, incorporated herein by reference, and are shown in Figure 6.

Marguerie *et al* (1992) *Immunology Today* **13**, 336-338, incorporated herein by reference, refers to PCR-based methods of analysing TCR mRNA.

Once the particular Vβ subset has been determined as herein described it is preferable to identify the combination of specific Vβ with specific J segment. This may be done, for example, by using a Vβ-specific oligonucleotide with an oligonucleotide specific for each J segment in a PCR reaction. Similarly, once a particular Vα subset has been determined as herein described the combination with a specific J segment can also be identified using PCR with Vα- and J-specific oligonucleotides.

It will also be appreciated that specific Vγ and specific Vδ segments may be identified, for example, by using oligonucleotides directed at specific Vγ segments in combination with Cγ-specific oligonucleotides, or by using oligonucleotides direct at specific Vδ segments in combination with Cδ-specific oligonucleotides. Combinations of specific Vγ and Vδ segments with specific J segments can be identified, for example, using substantially the same methods as for Vα-J and Vβ-J combinations by using suitable, selective oligonucleotides in a PCR reaction.

The number of specific T cell receptor-positive T cells or the number of T cell receptor-positive T cells in a specific subset may be determined using any suitable method. Conveniently, since many antibodies which are specific for specific Vβ segments and specific Vα segments of the TCR are available, the determination is made using antibodies which bind to a specific T cell receptor or to a specific subset of T cell receptors (for example to a specific Vβ segment of the T cell receptor).

Monoclonal antibodies directed at specific Vβ and Vα chains are readily available.

Endogen sells the following antibodies (distributed by Bradsure Biologicals Ltd, 67a Brook Street, Shepshed, Loughborough, Leics 9RF): Antibodies: Vβ3.1, Vβ5a, Vβ5c, Vβ6.7a, Vβ7.1, Vβ8, Vβ8b, Vβ12, Vβ13, Vβ17, Vα2, Vα12.1, Vγ4, Vγ9, Vδ1, Vδ2, δV1-J1.

Similarly, Immunotech sells the following antibodies (taken over by Coulter Electronics Ltd, Northwell Drive, Luton, Beds LU3 3RH): Antibodies: γδ minus Vδ1, Vδ2, Vδ3, Vγ1, Vγ9, Vα24, Vβ1, Vβ2, Vβ3, Vβ5.1, Vβ5.2, Vβ5.3, Vβ6.1, Vβ8.1 and Vβ8.2, Vβ9, Vβ11, Vβ12, Vβ13.1, Vβ13.6, Vβ14, Vβ16, Vβ17, Vβ18, Vβ20, Vβ21.3, Vβ22.1.

In addition, monoclonal antibodies against specific T cell receptors may be raised by immunising mice against specific human T cell tumour lines, making mouse B cell hybridomas and then screening the mouse antibodies produced against both the target cell line and different human T cell tumour lines using methods well known in the art. In this way, the hybridoma clones selected are likely to be producing anti-TCR specific monoclonal antibodies. A second approach is to substitute the V region on the β chain of a mouse TCR with a human Vβ region and using the cell line created to immunise mice. In this way, monoclonal antibodies directed at human Vβ chains may be generated and much of the background screening is eliminated and a greater range of targets could be generated. Since it is now possible to generate soluble human

TCRs, these may be used as targets for raising further monoclonal antibodies against Vβ and Vα targets using methods known in the art.

Quantitation of the number of specific T cell receptor-positive T cells can be done using well known methods. For example, the antibodies may be fluorescently labelled and the cells sorted and counted using a fluorescence-activated cell sorter (FACS) machine. Suitably, the antibodies are labelled with any convenient fluorescent compound, for example fluoroscein isothiocyanate (FITC).

Alternatively, but still preferably, the number of T cell receptor positive T cells or the number of T cell receptor-positive T cells in a specific subset may be determined by analysing the genomic DNA of the T cell population. Specific T cell DNA, which has been somatically rearranged, can be quantified in a similar manner to that of mRNA. Any method which will distinguish unrearranged and rearranged TCR genes may be used in order to determine the number of specific T cells.

Once the number of TCR mRNA which is specific for a T cell receptor or is specific for a subset of T cell receptors in the sample has been enumerated, and once the number of specific T cell receptor-positive T cells or specific T cell receptor-positive T cells of a particular subset has been enumerated, the number of specific TCR mRNA species per specific T cell is computed.

This is done for each of a sample containing T cells which have not responded to the antigen and a sample containing T cells which have responded to the antigen. The comparison may be made between the

test sample and a control sample wherein the control sample is an historic control sample or a sample taken contemporaneously from a separate, healthy individual or from a non-diseased site in the individual form which the test sample has been taken.

An increase in the amount of specific T cell receptor mRNA per specific T cell is indicative of that specific T cell (T cell receptor or subset of T cell receptors) being an antigen responsive T cell.

The increase in the amount of specific T cell receptor mRNA per specific T cell which is indicative of an antigen response varies depending on the particular antigen and the particular TCR or TCR subset.

Typically, an increase of greater than about 2 is indicative of a specific T cell response but the increase may be greater than 10 or greater than 100 and it may be greater than 1000.

As has been described previously, normal ranges of TCR gene expression may be determined for specific T cells or specific subsets of T cells. Preferably, an increase in the amount of specific T cell receptor mRNA per specific T cell is indicative of an antigen response if the increase is statistically significant by at least one, preferably at least two, and more preferably at least three or more standard deviations above the level of the control (ie the normal range of expression in the unstimulated situation).

The method is particularly useful to determine which T cell type is associated with a particular antigen-mediated disease. In some circumstances a predominant T cell type is involved in a disease process and, for example, the same T cell type is involved in the disease in the majority of individuals. Thus, the method is useful if in identifying the T cell type involved in the majority of individuals of a particular disease. However, the method is also particularly suited for use on individual patients in order to determine the specific T cell type involved in a particular disease in an individual patient. It will be appreciated that treatment of an individual patent may be tailored depending on the T cell type of the patient involved in the disease.

A further aspect of the invention provides a method of treating a patient wherein the patient has an antigen-mediated disease the method comprising (a) identifying an antigen-responsive T cell associated with an antigen-mediated disease according to the method of the first aspect of the invention and (b) administering to the patient an effective amount of an agent which ameliorates the disease.

The agent which ameliorates the disease is typically an agent which reduces or eliminates the T cell response to the antigen.

Once the identity of a specific T cell receptor or subset of T cell receptors has been determined as being involved in antigen-mediated disease using the method of the invention, an agent may be selected which ameliorates the disease. For example, monoclonal antibodies which are directed at a specific Vβ segment may be useful, or peptides which are derived from a CDR of a specific Vβ segment may be useful.

Experimental autoimmune encephalomyelitis (EAE), an animal model of the human condition multiple sclerosis, has provided a prototypic model to test the efficacy of anti-T cell therapy in autoimmune disease. In EAE in Lewis rats and PL/J mice, encephalitogenic T cells specific for myelin basic protein (MBP) were highly restricted, expressing similar TCRs that consisted of Vα2 and Vβ8.2 (Heber-Katz & Acha-Orbea, 1989) that could be successfully targeted for therapy by Vβ8.2-specific monoclonal antibodies. Moreover, vaccination with attenuated encephalitogenic T cells also mediated protection against EAE (Lider *et al*, 1988). Vaccination of rats with a peptide derived from the CDR2 (second complementarity determining region) of the Vβ8.2 TCR induced anti-idiotypic T cells and antibodies that inhibited the activation of pathogenic T cells and prevented and treated EAE (Vandenbark *et al*, 1989; Offner *et al*, 1991). Other studies have shown that TCR peptides derived from the CDR2 or other regions can induce immunoregulation of pathogenic T cells specific for MBP, collagen, heat shock protein, and the P2 protein of peripheral myelin, implicating a potential for therapy in experimental arthritis, neuritis in addition to EAE (Howell *et al*, 1989; Stevens *et al*, 1991; Kumar & Sercarz, 1993; Gregorian *et al*, 1993; Broeren *et al*, 1994; Matsumoto *et al*, 1994; Kuhrober *et al*, 1994; Haqqui *et al*, 1995).

Using a TCR-derived peptide as a vaccine in humans with progressive multiple sclerosis, a study has shown that vaccine responders have reduced immune reactivity and remain clinically stable over the course of one year of therapy, whereas non-responders continued to have

immune reactivity to MBP and deteriorated clinically (Vandenbark *et al*, 1996).

Methods of T cell vaccination, passive anti-T cell antibody therapy and peptide immunization are known. For example, see Heber-Katz E., Acha-Orbea H. (1989) *Immunol. Today* **10**, 164-169; Lider O., *et al* (1988) *Science* **239**, 181-183; Vandenbark A.A., *et al* (1989) *Nature* **341**, 541-544; Offner H., *et al* (1991) *Science* **251**, 430-432; Howell M.D., *et al* (1989) *Science* **246**, 668-670; Stevens D.B., *et al* (1991) *J. Neuroimmunol.* **37**, 123-129; Kumar V., Sercarz E.E. (1993) *J. Exp. Med.* **178**, 909-916; Gregorian S.K., *et al* (1993) *Am. Assoc. Immunol.* **150**, 28A; Broeren C.P.M., *et al* (1994) *Proc. Natl. Acad. Sci. USA.* **91**, 5997-6001; Matsumoto Y., *et al* (1994) *Cell. Immunol.* **153**, 468-478; Kuhrober A., *et al* (1994) *Eur. J. Immunol.* **24**, 1172-1180; and Haqqui T.M., *et al* (1995) *Ninth Int. Congr. Immunol. Abstr.* **5014**, 845, all incorporated herein by reference.

Thus, the invention also includes a method of selecting a treatment for a patient with an antigen-driven disease.

The invention will now be described in more detail with reference to the following Figures and Examples wherein:

Figure 1A + Figure 1B

Figure ~~1~~ shows a comparison of specificity of wild-type- and mutant-specific probes. Figure 1(a) shows a standard curve of amplified BV2S1 wt DNA; Figure 1(b) shows a standard curve of amplified BV2S1 mutant DNA.

Figure 2 shows the comparability of optical density ($OD_{450/630}$) readings obtained with wild-type- and mutant-specific probes. The ratio of ODs from wild-type and mutant probes on an αβ construct are shown.

Figure 3 describes the measurement of unknown cDNA samples. The quantitation of TCRBV2S1 cDNA is shown.

Figure 4 shows a comparison of *TCRBV2S1* mRNA production and CD25 expression by Vβ2.1 TCR^+ T cells in unseparated lymphocyte populations.

Figure 5 shows a comparison of *TCRBV3S1* mRNA production and CD25 expression by Vβ3.1 TCR^+ T cells in unseparated lymphocyte populations.

Figure 6 shows the sequence of PCR primers suitable for specific amplification of Vα and Vβ segments of TCR mRNA (cDNA). See Williams *et al* (1992) *J. Clin. Invest.* **90**, 326-333 for further details, incorporated herein by reference.

Figure 7 shows TCRBV mRNA levels per cell before and after 3 days culture in medium alone or supplemented with the superantigens SEB or TSST-1.

Figure 8 is a comparison of CD25 expression and intracellular TCRBV levels in antigen-triggered T cells.

Figure 9 shows intracellular TCRBV3S1 mRNA levels obtained from lymphocytes cultured with medium alone (UnRx) or supplemented with anti-CD28 (CD), anti-Vβ3.1 (Vβ) or a combination of the two (Vβ + CD).

Figure 10 shows the measurement of 15 000 molecules of a cloned TCRBV17S1 sequence on a single day (grouped) or over a period of 8 weeks (separate).

Figure 11 shows the accuracy of measurement of 15 000 molecules of 5 different TCRBV17S1 wild-type templates with a single mutant BV17S1 clone. Measurements were made using both a B17S1-specific primer and a TCRBC-specific primer (βPCR5′). There was no significant difference between the measurements obtained using either of these primers.

Figure 12 shows the measurement of wild-type BV17S1 (clone 7) and mutant BV17S1 (17/2/17 mutant) template using BV17S1-specific and βPCR5′ primers.

Figure 13 shows the measurement of BV17S1 wild-type templates (clone 1 and clone 7) with their respective mutants.

Figure 14 shows the measurement of different numbers of molecules of wild-type TCRBV17S1 template.

Figure 15 shows the relative activities different promoter - pXP2 constructs transfected into Jurkat T cells after 40 hours culture in medium alone (●) or supplemented with PMA (○).

Example 1: Identification of antigen-responsive T cells

I propose that TCR gene expression is increased after antigen stimulation in order to replace the receptors which have been lost from the cell surface during antigen triggering. As published data suggests that almost half of the cell surface TCRs are required to bind antigen (and

are therefore phosphorylated and internalised) to commit the T cell to full activation [46], I propose that this mechanism would be vital for normal T cell function.

As T cell activation by cytokines would not involve degradation of cell-surface TCRs, I propose that measurement of TCR-specific mRNA production rates is a viable method to discriminate between passively recruited/passively-activated T cells from antigen-specific T cell effectors in any immune process.

T cell receptor (TCR) messenger RNA (mRNA) is measured by a reverse transcription competitive polymerase chain reaction (RT-CPCR) (see, for example, Kohsaka *et al* (1993) *Nucl. Acids Res.* **21**, 3469-3472; and Taniguchi *et al* (1994) *J. Immunol. Methods* **169**, 101-109. In this method, a mutant template is added at different concentrations to aliquots of wild-type cDNA template and the ratio of the two is determined by mutant and wild-type-specific oligonucleotide probes after PCR. Quantification is expressed as the number of molecules of specific TCR mRNA expressed per specific TCR + ve T cell. The increase in gene transcription levels between unstimulated and antigen-triggered cells can be between 2- and 1000-fold. This varies for different *TCRBV* genes. Our data suggests that SEB-driven *TCRBV3S1* transcription is increased approximately 30-fold over control cultures in the presence of medium alone [range 8.7- to 161.7-fold] but only 6.7-fold [range 3.7- to 8.4-fold] over cultures in the presence of TSST-1. We do not know whether this represents a true picture of background triggering or reflects either contaminants in the TSST-1 preparation or TCR α chain-binding by TSST-1. *TCRBV2S1* increases 27-fold on average [range

8.3- to 349.8-fold] when specifically triggered by TSST-1. There appears to be no difference in *TCRBV2S1* transcription in control cultures of medium alone or the presence of SEB. Once transcription levels are known for each TCRBV gene, multiple antigen-triggered T cells are identifiable within any lesion in any condition. This is unlikely to be the case, if specific TCRBV mRNA levels are expressed as a percentage of total αβ TCR chain mRNA.

TCRBV gene mutants for use in RT-CPCR

TCRBV gene mutants are manufactured by cloning amplified, wild-type PCR products into a vector such as PCRscript according to manufacturer's instructions and mutating this cloned template. The mutation is performed using a PCR-based method known as gene SOEing (sequence overlap extension) according to the methods of Higuchi *et al* (1988) and Ho *et al* (1989). Briefly, the wild-type sequence is amplified in two halves, in separate reactions. The first reaction amplifies the upstream half of the template using the upstream *TCRBV*-specific primer and a mutational downstream primer. The mutational primer anneals to the template just upstream of the site to be mutated and carries 12 to 15 bp of the new mutation sequence at its 5′ end. The second reaction, which is carried out separately, amplifies the lower half of the template. It utilises the downstream *TCRBC*-specific primer and an upstream mutational primer. The upstream mutational primer anneals to the template just downstream of the site to be mutated and carries 12 to 15 bp of the new mutation sequence at its 5′ end. After amplification, each half is purified free of wild-type template, primers and Taq polymerase and diluted to be at equal concentration.

The two halves are then placed together in a PCR mix along with the *TCRBV*- and *TCRBC*-specific primers only and amplified in a 'hot-started' PCR reaction. During this second round of amplification, the 2 halves are annealed together by virtue of their overlapping mutation sequence and a new mutant template is created by the PCR process. The new PCR product can then be re-cloned back into a vector such as PCRscript and sequenced until an error-free clone is identified. Using this process we have replaced the CATCAGAAGCAGAGATCTCC sequence in the wild-type *TCRBC* region with the GATGTCAAGCTGGTCGAGAA sequence from the corresponding region of the *TCRAC* gene. This mutation was designed not to affect the overall size, dG/dC:dA/dT content or the primer annealing sequences of the original wild-type template. The mutated template amplifies with equal efficiency as the wild type template in a number of *TCRBV*-specific PCR reactions. See Higuchi R., *et al* (1988) *Nucleic Acids Res.* **15**, 7351-7367 and Ho S.N., *et al* (1989) *Gene* **77**, 51-9, for further details on gene SOEing.

Cell culture

Lymphocytes were obtained from a peripheral blood sample from a normal donor and cultured for three days at 1 x 10^6 cells/ml *in vitro* in RPMI 1640 with penicillin, streptomycin, glutamine and 10% (v/v) heat-inactivated foetal calf serum. Cells were incubated in this medium alone or medium supplemented with either 100 ng/ml *Staphylococcal enterotoxin B* (SEB a superantigen which binds Vβ3+ cells amongst others but not Vβ2+ T cells) or 10 ng/ml TSST-1 (which binds Vβ2+ T cells but not Vβ3+ ones) [51].

Measurement of specific TCR mRNA

Total RNA is extracted from T cells either *in vivo* or *in vitro* using the RNeasy™ extraction kit according to manufacturer's instructions (Qiagen) following cell lysis and DNA shearing by the Qiashredder™ (Qiagen). DNA contamination may be removed by treatment with RNase-deficient DNase for 1 hr at 37°C followed by heat inactivation of the enzyme by incubation at 75°C for 5 mins. Total RNA concentrations may be sometimes measured at this point but are often less than can be quantified by spectrometry. 9.5 µl of the total RNA is then reverse transcribed with 100 U M-MLV reverse transcriptase (Superscript^{II}; Life Technologies) in a final volume of 20 µl for 90 min following a 'hot start' according to manufacturer's instructions. One µl of cDNA is amplified with 0.1x, 1x and 10x molecules of mutant plasmid DNA. Estimations of cDNA concentration can be made by amplification of 1 µl of cDNA against a standard curve of cloned specific-TCRBV gene product of known concentration.

The PCR reaction is in 50 µl final volume using 1 U Red Hot™ Taq polymerase (Advanced Biotechnologies) with between 10 and 25 pMol each of a TCRBV-specific and an aminated-TCRBC-specific oligonucleotide primer, 200 µM dNTPs and 2.0 mM $MgCl_2$. The reaction is 'hot started' at 95°C for 10 min, and cooled to 25°C at a rate of 3°C per min on a PHC-3 thermal cycler (Techne). When at 25°C, 1 U of Red Hot™ Taq polymerase is added and the reaction is extended at 72°C for 3 min followed by 35 cycles of 95°C for 1 min, 54-56°C for 30 sec and 72°C for 30 sec with a final extension for 5 min at 72°C.

Five µl are run on a 1.5% (w/v) agarose gel to confirm that the PCR has worked. Ratified sequences for TCRBV-specific oligomers have been published for the first 20 TCRBV families [Williams *et al* (1992) *J. Clin. Invest.* **90**, 326-333, incorporated herein by reference]. The additional, functional, 5 or so (according to classification) functional TCRBV gene families can be amplified by designing primers to their published sequence [Rowen *et al* (1996) *Science* **272**, 1755-1762, incorporated herein by reference]. The sequence and its annotation are deposited in the Genome Sequence Data Base (accession numbers L36092, L36190 and U03115). Usually primers are designed which anneal to the CDR1 or CDR2 regions of the gene as these are the areas most likely to differ from other TCRBV sequences. The amplified PCR products are separated from the unincorporated primers using the 'clean up' kit (Advanced Biotechnologies Ltd) according to the manufacturer's instructions. The purified PCR product is eluted in 90 µl H_2O and mixed with an equal volume of MES/EDTA buffer (50 mM (2-[N-morpholino]ethanesulfonic acid), 1 mM EDTA). Forty µl of this mixture are added to each of four wells of a covalent ELISA plate (2388; Corning Costar) along with 40 µl of a cross-linking solution (40 mg EDC and 0.543 mg sulfo-NHS in 5 mls of H_2O) and incubated overnight at 37°C. The plate is washed 3X with PBS pH 7.4. The bound DNA is denatured with 100 µl 0.1 M NaOH for 10 min at RT. After discarding the NaOH, the plate is washed with once, 0.1X SSC, twice with HW buffer (6X SSC + 0.1% (v/v) n-lauroylsarcosine). The plate is then blocked with 5% Marvel in HW buffer for 30 min at 37°C.

The single-stranded DNA bound to the plate is probed either with a Biotin-conjugated, wild-type-specific (2 wells) or a Biotin-conjugated,

mutant-specific (2 wells) oligonucleotide dissolved in HW for 90 min at 42°C. The plates are then washed three times with HW2 buffer (2XSSC + 0.1% (v/v) n-lauroylsarcosine) and once with buffer B (100 mM Tris-HCl pH 7.5 + 800 mM NaCl + 0.5% (w/v) Blocking reagent (Boehringer Mannheim)). The bound probes are then detected by incubating the wells for 1 hr at 37°C with 100 µl ABC (Avidin Biotin Complex) streptavidin peroxidase (Dako Ltd) made up according to the manufacturer's instructions and diluted 1:10000 in Buffer B. The plate is then washed 1X with Buffer B and 5X with Buffer A (100 mM Tris-HCl pH 7.5 and 800 mM NaCl). Peroxidase activity was detected by adding a 100 µl of TMB substrate (1 mg/ml) in a phosphate-citrate buffer with 1 µl H_2O_2. When the appropriate colour density has developed the reaction is stopped with 100 µl of a 1M H_2SO_4 solution and read on a dual wavelength ELISA reader at 450 nM with correction at 630 nM.

The amount of mutant and wild-type product in each reaction is directly in proportion to their optical densities. Plotting $\log_{10}$ optical density (450/630) against $\log_{10}$ initial mutant reaction concentration allows derivation of initial wild-type cDNA concentration. The point where the $\log_{10}$ optical density ratio is 0 is the point where the two templates were at the same initial concentration.

The comparability of the optical densities derived from mutant- and wild-type-specific oligonucleotide probes was determined by constructing a template which contained one copy of each of these sequences contained within a genetic region spanned by the *TCRBV2S1* and *TCRBC* PCR primer annealing sequences. This construct was

manufactured by restricting wild-type and mutant clones with *Hpa*I and *Bal*I. The appropriate fragments were ligated together, and re-amplified using the *TCRBV2S1* and *TCRBC*-specific primers. The new PCR product was cloned into the PCRscript vector. This template was sequenced to ensure it contained error-free annealing sites for the *TCRBV2S1*, *TCRBC*, wild-type and mutant sequences. This template was then amplified using a biotinylated *TCRBC*-specific primer, and bound at various dilutions to a streptavidin-coated plate. The line comparing the optical densities obtained with the two probes over a variety of amplified template dilutions was a straight line ($r^2 = 1.00$) with a gradient of 0.99 ± 0.02 going through the origin at 0.00, 0.00.

If a specific TCR β chain sequence is to be quantified this is performed using the above method using either a specific TCRBV-TCRBJ combination of oligonucleotide primers or a specific TCRBV-N region combination of oligonucleotide primers. In either case, the mutation is best placed within the TCRBV sequence. It can be performed by gene SOEing as before and the most convenient mutation is to replace a stretch of sense sequence with antisense sequence. This leaves the G/C:A/T content the same and it is certain that both mutant and wild-type probes will have equal affinity. The PCR annealing site which is furthest away from the probe target sequence should be used as the end whose PCR oligomer is biotinylated.

Determining the number of specific Vβ+ T cells

As the calculation of T cell activation requires that the amount of specific mRNA per specific Vβ+ T cell is known, both the specific

Vβ+ T cell numbers and the amounts of TCRBV-specific mRNA molecules are enumerated. Specific T cell numbers is measured by using a combination of Vβ-specific monoclonal antibodies and cell counting.

TCR and CD25 Immunohistochemistry: Aliquots of 5 x 10^5 cells were pelleted and resuspended in 50 ml PBS pH 7.6 with 0.01% (w/v) sodium azide and 10% (v/v) heat inactivated normal human serum. These were incubated with 20 ml FITC-conjugated anti-Vβ2, anti-Vβ3 or anti-CD3 and counter stained with 10 ml RPE-conjugated anti-CD25 for 40 min on ice. Cells were then washed x 3 and fixed in 500 ml of PBS with 0.5% (v/v) paraformaldehyde buffered to pH 7.4. Samples were processed on a FACScan analyser (Beckton Dickinson).

In the absence of a Vβ-specific monoclonal antibody being available, specific TCRBV-containing DNA can be quantified. Specific T cell DNA, which has been somatically re-arranged, can be quantified as for specific β chain mRNA as CPCR. Once more, a TCRBV-specific oligomer is used in combination with TCRBJ-specific primers covering all the thirteen TCRBJ sequences known to occur. The mutation, as above, should be placed in the TCRBV sequence. Once numbers of DNA molecules are known for each TCRBJ combination the numbers of T cells can be calculated as each T cell should contain only one BV-BJ combination. Sometimes it is valuable to pre-restrict genomic DNA before amplification with an enzyme which cuts between the TCRBJ element you wish to amplify from and the one behind it. Slight overestimations in T cell number may occur using this method because γδ T cells may also have a re-arranged TCRB gene complex.

Alternatively, as the TCRBJ segments BJ1S1, BJ2S1 and BJ2S7 seem to be the most frequently used, measuring the TCRBV-BJ combinations for just these three segments will approximate to 40-45% of the total Vβ-specific T cell gene rearrangements for most Vβs [Jeddi-Tehrani *et al* (1994) *Human Immunology* **40**, 93-100]. Quantitation of TCRA mRNA, total and specific, may also be achieved using the RT-CPCR method. In the first instance, TCRA mutants have to be manufactured. In the reverse of the TCRB method, mutants have the GATGTCAAGCTGGTCGAGAA wild-type TCRAC sequence replaced with the equivalent sequence from the TCRBC chain, CATCAGAAGCAGAGATCTCC by gene SOEing. This may be performed to sequences derived using *TCRAV*- and *TCRAC*-specific primers (for quantification of specific α chain message) or in sequences amplified using two *TCRAC*-specific primers (for quantitation of total αβ mRNA).

Otherwise the method is as described for specific TCRBV cDNA measurement. Measurement of total αβ mRNA levels using TCRAC-specific primers is by far superior to quantifying the levels of TCRBC mRNA. This is because TCR β chain mRNA is also made by γδ T cells whereas TCR α chain mRNA is not.

If specific TCRAV-TCRAJ combinations are to be measured, the method is as for TCRBV-TRCBJ quantitation. Namely, the mutation is placed in the TCRAV region and is conveniently the antisense of the existing sequence. Either, TCRAV- or TCRAJ-specific primers may be biotinylated depending on which is further from the oligomer probe annealing site.

Results are shown in Figures 1 to 5.

Figure 1 shows a comparison of specificity of wild-type- and mutant-specific probes. A *TCRBV2S1* mRNA mRNA transcript was reverse transcribed, amplified by PCR and cloned into the pBluescript vector. It was mutated by gene SOEing so that a 20 bp sequence in the *TCRBC* region was replaced with a 20 bp sequence from the corresponding region of the *TCRAC* gene. The mutant was also cloned into pBluescript. Varying numbers of molecules of wild-type (wt) and mutant (mut) sequence (from 10 to 10^7 molecules) were amplified by PCR using *TCRBV2S1*- and *TCRBC*-specific primers. The amplified templates were attached to an amine-binding plate by virtue of the aminated-group added to the 5′ end of the TCRBC-specific primer according to the method described earlier. Figure 1 shows the results of probing wt and mut amplicons with both wt- and mut-specific probes.

Each probe appeared to be completely specific for its target sequence, there appeared to be no cross-reactivity over a 10^6-fold range of initial template number.

Figure 2 shows the comparability of optical density ($OD_{450/630}$) readings obtained with wild-type- and mutant-specific probes. A construct containing both wild-type and mutant sequences was manufactured (see above) and cloned. Different numbers of constructs (0 to 10^6 molecules) were amplified with *TCRBV2S1*- and *TCRBC*-specific primers, attached to an amine binding ELISA plate and then probed with wild-type- and mutant-specific probes (as described above).

The ODs obtained from each probe against the construct target were precisely comparable over the range 0 to 10^6 molecules.

Figure 3 describes the measurement of unknown cDNA samples. Aliquots of an unknown quantity of *TCRBV2S1* cDNA are mixed with varying known amounts of mut *TCRBV2S1* and the two are co-amplified in a series of PCR reactions. After having been assayed using the DNA capture ELISA, the ratios of mutant to wild-type amplicons are plotted against the starting mutant template concentration. The point where the log of the ratio is 0 (ie the ratio of mutant to wild-type amplicon is 1) is the point where wild-type and mutant templates were present at the same initial concentration.

Figure 4 shows a comparison of *TCRBV2S1* mRNA production and CD25 expression by Vβ2.1 TCR^+ T cells in unseparated lymphocyte populations. The Vβ2.1 T cells, within a population of unseparated peripheral blood lymphocytes, were analysed for *TCRBV2S1* mRNA production (molecules per cell) and CD25 expression (% positivity). The analyses took place prior to culture (PRE) and three days after being cultured in the presence of medium alone (CON), or medium supplemented with either 100 ng/ml staphylococcal enterotoxin B (SEB) or 10 ng/ml toxic shock syndrome toxin-1 (TSST-1). Four normal individuals were examined.

TCRBV2S1 production was much higher in the appropriately stimulated (TSST-1) cell cultures than with any of the controls. The magnitude of increase in mRNA production was much larger than that seen in CD25 positivity suggesting that analysis of specific TCR mRNA production

rates gives the clearest indication of the antigen-driven T cells within an unseparated lymphocyte population.

Figure 5 is a comparison of *TCRBV3S1* mRNA production and CD25 expression by Vβ3.1 TCR^+ T cells in unseparated lymphocyte populations. The Vβ3.1 T cells, within a population of unseparated peripheral blood lymphocytes, were analysed for *TCRBV3S1* mRNA production (molecules per cell) and CD25 expression (% positivity). The analyses took place prior to culture (PRE) and three days after being cultured in the presence of medium alone (CON), or medium supplemented with either 100 ng/ml staphylococcal enterotoxin B (SEB) or 10 ng/ml toxic shock syndrome toxin-1 (TSST-1). Four normal individuals were examined.

The levels of TCRBV3S1 mRNA production were highest in the appropriately stimulated lymphocytes (SEB). An increased amount of TCRBV3S1 was seen in TSST-1 stimulated cultures compared with PRE and CON controls. This was still significantly less than that observed in SEB-stimulated cultures. There is no significant difference in the CD25 expression by the Vβ3.1 T cells in the TSST-1 and SEB cultures. This confirms that analysis of specific mRNA production per specific T cell provides the clearest indication of identity of the antigen-driven T cells within unseparated lymphocyte populations.

Conclusions

There are a number of conclusions suggested by these data.

1. The constitutive expression of different TCR genes vary. Control cultures of Vβ2.1+ T cells produce fewer molecules/T cell than Vβ3.1+ T cells in the same cultures.

2. TCR production rates increase greatly when T cells are stimulated by their specific antigen but the magnitude of this increase varies for different TCR genes.

3. TCR mRNA molecules per cell are increased in passive cultures with activated lymphocytes but not to the same degree as when the T cell is directly stimulated with antigen.

4. CD25+ expression appears to be independently regulated from TCR gene transcription.

5. Taken together, these data suggest that measurement of specific TCR mRNA amount of mRNA per cell may discriminate between direct antigenic triggering of T cells and passive, cytokine-mediated activation in immune lesions.

Example 2: Graft-versus-Host disease in a Rat model

This is an example of measuring TCR mRNA molecules per T cell as an index of antigen-mediated TCR triggering *in vivo*.

Recent evidence has shown that long-term T cell lines and clones from DA rats generated against irradiated Lewis rat lymphocyte targets use a TCR encoded by the *TCRBV6S1* gene segment recombined with the

TCRBJ2S1 gene segment and bearing a distinct N region sequence almost exclusively (Tavakol Afshari *et al* (1997) *Transpl. Immunol.*, in press). A number of DA and DA X Lewis F_1 rats are obtained and injected with unseparated DA lymphocytes into their hind footpads. Over a period of 14 days draining lymph nodes are obtained from injected rats, the DA lymphocytes are purified by eliminating $RT1^l$ cell surface marker-positive lymphocytes (F_1 cells) and the numbers of *TCRBV6S1-TCRBJ2S1* mRNA and genomic DNA molecules are measured essentially as described in Example 1. The DA lymphocytes obtained from DA X Lewis have higher *TCRBV6S1-TCRBJ2S1* mRNA to DNA ratios (indicating higher levels of specific TCR mRNA per specific T cell) than those obtained from control (DA) animals.

Example 3: Measurement of levels of *TCRBV13S2* mRNA per Vβ13.2 TCR-positive T cell in Hypergammaglobulinaemic Primary Sjögren's Syndrome (HGPSS)

HGPSS is an autoimmune disorder characterised by T cell infiltration with and immune destruction of lacrimal and salivary glands. There is evidence of a genetic susceptibility to this condition encoded by the *TCRBV13S2* gene and elevated levels of TCRBV13 mRNA in the salivary glands of patients with this condition (Kay *et al*, 1995; Sumida *et al*, 1992). Biopsies of minor salivary glands and peripheral blood samples are obtained from patients with this condition. Minor salivary gland biopsies from patients undergoing dental surgery for unrelated conditions (impacted wisdom teeth removal) are also obtained. The biopsies are collagenase digested. The lymphocytes from digested biopsies and the peripheral blood samples are purified by separation

through a Ficoll gradient and the Vβ13.2+ T cell numbers are quantified by a combination of white cell counts and FACS analysis following immunohistochemical staining using an anti-Vβ13.2, FITC-conjugated monoclonal antibody. The *TCRBV13S2* mRNA levels are measured as described in Example 1. The levels of *TCRBV13S2* mRNA per specific Vβ13.2+ T cell are higher in the HGPSS patients' salivary glands than in the HGPSS patients' peripheral blood (selective homing to and triggering within diseased tissue) or in the salivary glands of patients with irrelevant dental disorders. (See Kay R.A., Hutchings C.J., Ollier W.E.R. (1995) *Human Immunol.* **42**, 328-330 and Sumida T., *et al.* (1992) *J. Clin. Invest.* **89**, 681-685.)

Example 4: Response to superantigenic stimulation in four unrelated individuals

Lymphocytes were separated from the peripheral blood samples from 4 normal donors and cultured for 3 days at 1.5 x 10^6 cells/ml in RPMI 1640 with penicillin, streptomycin, glutamine and 10% (v/v) heat-inactivated foetal calf serum. Cells were incubated in medium alone or medium supplemented with either 100 ng/ml SEB or 10 ng/ml TSST-1. Messenger RNA levels were measured as using the contaminant reverse-transcription PCR as described previously in Example 1. The numbers of $V\beta2.1^+$ and $V\beta3.1^+$ cells along with their CD25 status was performed by FACS analysis as described earlier in Example 1. Measurements were made prior to (PRE) as well as after 3 days culture.

The results confirm that SEB selectively stimulates *TCRBV3S1* levels in $V\beta3.1^+$ T cells and that TSST-1 does the same for *TCRBV2S1* levels in

Vβ2.1$^+$ T cells. The relative increase in mRNA levels was 58-fold for *BV3S1* and 40-fold for *BV2S1* (Figure 7). Unstimulated TCR mRNA levels per cell higher for *BV3S1* than *BV2S1* suggesting that even at rest the *TCRBV3S1* gene is transcribed at a higher level. Figure 7 shows *TCRBV* mRNA levels per cell before and after 3 days culture in medium alone or supplemented with the superantigens SEB or TSST-1.

As before the results also show that *TCRBV* levels per cell are a better indicator of antigen-specific T cell triggering than the measurement of CD25 expression (Figure 8). Figure 8 is a comparison of CD25 expression and intracellular *TCRBV* levels in antigen-triggered T cells.

Example 5: T cell response to stimulation with conventional antigen

Lymphocytes were separated from a peripheral blood sample and cultured as before for 3 days in medium alone or medium supplemented with an anti-Vβ3.1-specific monoclonal antibody, an anti-CD28-specific monoclonal antibody or both antibodies in combination.

Anti-CD28 alone did not significantly increase intracellular *BV3S1* mRNA levels. Anti-Vβ3.1 did significantly increase intracellular *BV3S1* levels. However, intracellular *BV3S1* mRNA levels comparable to those obtained with SEB were only obtained after 3 days culture with both anti-CD28 and anti-Vβ3.1 (Figure 9). Figure 9 shows the intracellular *TCRBV3S1* mRNA levels obtained from lymphocytes cultured with medium alone (UnRx) or supplemented with anti-CD28 (CD), anti-Vβ3.1 (Vβ) or a combination of the two (Vβ + CD).

Using an anti-Vβ-specific monoclonal antibody is equivalent to stimulation with conventional antigen. This experiment demonstrates that this method is suitable for use in situations where conventional antigen (as well as superantigen) might be thought to be responsible for T cell stimulation. It also demonstrates that CD28 co-stimulation is as important for full TCR gene transcription as it is for T cell proliferation.

Example 6: Assay performance characteristics

In order to investigate the stability of the assay over time, a *TCRBV17S1*-specific mRNA was reverse transcribed, amplified, cloned and sequenced. The clone was mutated, as previously described, in the *TCRBC* region so that it contained a short *TCRAC* sequence and could be used as a measurement contaminant for the wild-type template.

Fifteen thousand molecules of wild-type template were measured either on 1 day or over a period of 8 weeks using the contaminant PCR method. The results are shown in Figure 10 and demonstrate that no significant drift in the assay's accuracy occurred over this period of time.

Figure 10 shows the measurement of 15,000 molecules of a cloned *TCRBV17S1* sequence on a single day (grouped) or over a period of 8 weeks (separate).

The variable domains of the β chain of the TCR are encoded by a combination of 3 smaller gene segments. Therefore in each different *TCRBV17S1*-encoded TCR, the *TCRBV17S1* gene segment will be

combined with a different *BD* and *BJ* gene segment. In order to see if this physiological variation affects the accuracy of measurement, a mutant *BV17S1* clone (using *BD1* and *BJ1S1*) was used to measure 5 different *BV17S1* wild-type templates. One of these templates had the same *BD* and *BJ* combination as the mutant; the other 4 did not. If these different segments did affect measurement, one could check this by measuring the wild types using a primer that annealed purely to the *TCRBC* region, omitting the upstream areas of genetic variation. Accordingly, Figure 11 shows the results of measuring these 5 different *BV17S1* clones, with a single mutant contaminant using both a *BV17S1*-specific primer (which includes the regions of genetic variability) and a βPCR5′ primer (which excludes the regions of genetic variability).

The results show that the genetic variation that naturally occurs in TCR gene rearrangement does not affect the ability of a single mutant clone to provide accurate data. Furthermore, a single mutant clone can measure a polyclonal population of TCR sequences or a single TCR template with equal efficiency (data not shown).

Figure 11 shows the accuracy of measurement of 15,000 molecules of 5 different *TCRBV17S1* wild-type templates with a single mutant *BV17S1* clone. Measurements were made using both a *BV17S1*-specific primer and a *TCRBC*-specific primer (βPCR5′) were used. There was no significant difference between the measurements obtained using either of these primers.

In order to see if un-physiological variation in genetic sequence interfered with this test, a mutant was manufactured from one of the

BV17S1 wild-type templates. This mutant was identical to the wild-type in all but one respect. A short sequence, approximately 60 bp long, was substituted from the middle of the *BV2S1* variable gene into the middle of the *BV17S1* sequence (17/2/17 mutant). This altered sequence homology between this mutant and the wild-type by no more than naturally occurs between different wild-types with *BD/BJ* substitution. Measurements were made of the two templates using both the *BV17S1*-specific primer (which anneals 5′ to the mutation) and βPCR5′ primer (which anneals downstream to the mutation). The results are shown in Figure 12. Figure 12 shows the measurement of wild-type *BV17S1* (clone 7) and mutant *BV17S1* (17/2/17 mutant) template using *BV17S1*-specific and βPCR5′ primers.

The results show that the substitution of part of the *BV17S1* sequence with an analogous section of *BV2S1* (which would never occur naturally) abrogates the measurement of the mutant template with a primer which anneals upstream of the mutation. If a primer is used which anneals downstream of this mutation (βPCR5′), then there is no significant difference between the accuracy of measurement of the wild-type or mutant templates.

In order to see whether the accurate measurement of a range of wild-type templates by one mutant was the peculiar characteristic of that mutant, a second measuring mutant was made using a different *BV17S1* wild-type template as a starting point. The results are shown in Figure 13 and demonstrate that either mutant is capable of accurately measuring both wild-type templates. Figure 13 shows the measurement of *BV17S1* wild-type templates (clone 1 and clone 7) with their respective mutants.

Finally, the assay was assessed in its ability to measure a range of wild-type molecule numbers. One hundred thousand, 10,000 and 1,000 molecules of *TCRBV17S1* template were measured (Figure 14). The inter-assay coefficient of variation was comparable over this range of molecule numbers and was approximately 23%. The test easily distinguished a ten-fold difference in template numbers. The measurements were designed to cover a range that exceeded, in both directions, any molecule numbers which have so far been encountered in the experiments *in vitro*.

Figure 14 shows the measurement of different numbers of molecules of wild-type *TCRBV17S1* template.

In summary, the contaminant, quantitative PCR method is capable of measuring the numbers of TCR mRNA molecules likely to be encountered *in vitro* (and probably *in vivo*) with an accuracy of about 23%. As intracellular TCR mRNA levels change about 40-fold after appropriate stimulation, this test should (and does) easily detect T cell triggering. A single mutant template for each *TCRBV* gene is all that is necessary to measure monoclonal, oligoclonal and polyclonal TCR mRNA populations and the accuracy of the assay does not appear to drift with time.

Example 7: TCRBV gene promoter function at rest and after activation

Data from Example 1 shows that intracellular mRNA levels varied between different *TCRBV* gene both at rest and after activation. Given the sequence differences that occur between individual *TCRBV* gene promoters it seems likely that this represents differences in gene transcription.

In order to test this, rat *TCRBV* promoters were isolated from genomic DNA by PCR cloned into the pGEM-T Easy vector (Promega), and sequenced. These promoters from different *TCRBV* genes were subcloned into the luciferase reporter gene vector, pXP2. These constructs were transfected by electroporation along with a co-transfectant control (a thymidine kinase promoter controlling renilla luciferase expression) into the human T cell line, Jurkat. The cells were cultured for 40 hours after transfection either in medium alone or medium supplemented with PMA at 10 ng/ml. The PMA pharmacologically mimics TCR triggering. After 40 hours, the cells were harvested and lysed. Firefly luciferase (*TCRBV* gene-controlled) and renilla luciferase measurements were made using the dual luciferase assay kit (Promega) and the ratios are shown in Figure 15.

These data strongly suggest that different *TCRBV* genes (even ones in the same family such as *BV5S1* and *BV5S2*) are transcribed at different rates from each other both at rest and after activation. These data appear to confirm the earlier suggestions that differences in intracellular TCR mRNA levels might reflect differences in *TCRBV*-specific mRNA

production. It should be noted that the magnitude of the differences seen in intracellular mRNA levels is greater than those observed with the reporter constructs. This is probably due to not including the *TCRB* enhancer in the reporter constructs. The *TCRB* enhancer is relatively very strong compared to the promoter and increases luciferase production markedly in transfection experiments. Figure 15 shows the relative activities different promoter-pXP2 constructs transfected into Jurkat T cells after 40 hours culture in medium alone (●) or supplemented with PMA (○).

The information provided in Examples 4 to 7

(1) shows that TCR mRNA levels per cell increase specifically in response to TSST-1 and SEB in 4 further, unrelated individuals;

(2) confirms that intracellular TCR mRNA levels are a better discriminator of antigen stimulation than CD25 expression;

(3) shows that a similar rise in the level of TCR mRNA can be induced by conventional antigen stimulation (approximated to by a Vβ-specific monoclonal antibody) but only when the T cell is co-stimulated by anti-CD28;

(4) demonstrates that the quantitative PCR method is accurate (with an inter-assay variation of approximately 25% or less), is stable over time and is unaffected by the physiological sequence variation that occurs in TCR genes. However, the assay is sensitive to un-physiological gene mutations; and

(5) shows luciferase reporter gene assay data using rat TCR gene promoters transfected in human Jurkat T cell lines which demonstrate that TCR gene transcription is increased after T cell stimulation and does vary, both at rest and after stimulation, with different *TCRBV* genes.

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